NAT2 slow acetylation, *GSTM1* null genotype, and risk of bladder cancer: results from the Spanish Bladder Cancer Study and meta-analyses

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Summary

Background **Many reported associations between common genetic polymorphisms and complex diseases have not been confirmed in subsequent studies. An exception could be the association between *NAT2* slow acetylation, *GSTM1* null genotype, and bladder-cancer risk. However, current evidence is based on meta-analyses of relatively small studies (range 23–374 cases) with some evidence of publication bias and study heterogeneity. Associations between polymorphisms in other *NAT* and *GST* genes and bladder-cancer risk have been inconsistent.**

Methods **We investigated polymorphisms in *NAT2*, *GSTM1*, *NAT1*, *GSTT1*, *GSTM3*, and *GSTP1* in 1150 patients with transitional-cell carcinoma of the urinary bladder and 1149 controls in Spain; all the participants were white. We also carried out meta-analyses of *NAT2*, *GSTM1*, and bladder cancer that included more than twice as many cases as in previous reports.**

Findings **In our study, the odds ratios for bladder cancer for individuals with deletion of one or two copies of the *GSTM1* gene were 1·2 (95% CI 0·8–1·7) and 1·9 (1·4–2·7) respectively (p for trend <0·0001). Compared with *NAT2* rapid or intermediate acetylators, *NAT2* slow acetylators had an increased overall risk of bladder cancer (1·4 [1·2–1·7]) that was stronger for cigarette smokers than for never smokers (p for interaction 0·008). No significant associations were found with the other polymorphisms. Meta-analyses showed that the overall association for *NAT2* was robust (p<0·0001), and case-only meta-analyses provided support for an interaction between *NAT2* and smoking (p for interaction 0·009). The overall association for *GSTM1* was also robust (p<0·0001) and was not modified by smoking status (p=0·86).**

Interpretation **The *GSTM1* null genotype increases the overall risk of bladder cancer, and the *NAT2* slow-acetylator genotype increases risk particularly among cigarette smokers. These findings provide compelling evidence for the role of common polymorphisms in the aetiology of cancer.**

Relevance to practice **Although the relative risks are modest, these polymorphisms could account for up to 31% of bladder cancers because of their high prevalence.**

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See Comment page 610

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Introduction

The inability to replicate results on many associations between common genetic polymorphisms and complex diseases has raised scepticism in this area of research.[1] One of the few exceptions could be the association between the risk of bladder cancer and polymorphisms in two carcinogen-detoxification genes—*NAT2* and *GSTM1*. However, evidence for an association relies on analyses of pooled data and meta-analyses of relatively small studies (range 23–374 patients, average about 100 per study), and concern has been raised about publication bias and heterogeneity of results.[2–9] Tobacco smoking is an important cause of bladder cancer,[10] and previous analyses have suggested that the relative risk from smoking is stronger for *NAT2* slow acetylators than for rapid or intermediate acetylators.[2,5,11] This interaction is biologically plausible, since aromatic amines, which are thought to be the most important class of bladder carcinogens in tobacco smoke,[12] are detoxified by *NAT2*.[13] However, epidemiological evidence for this interaction is even weaker than for the overall genotype association. Associations between bladder-cancer risk and polymorphisms in other carcinogen-detoxification genes such as *NAT1* and other glutathione-S-transferases have been less frequently explored, with inconsistent results across studies.[14–35]

We report results on the associations of polymorphisms in *NAT* and *GST* genes with bladder-cancer risk and their interaction with cigarette smoking among participants in the Spanish Bladder Cancer Study. This study was designed to have adequate statistical power for rigorous evaluation of the proposed associations between genetic variation in *NAT2* and *GSTM1* and bladder-cancer risk. We also report meta-analyses of *NAT2*, *GSTM1*, smoking, and bladder cancer that include more than twice as many patients as in previous reports.

	Cases (n=1150)	Controls (n=1149)
Demography		
Mean age (SD), years	66 (10)	65 (10)
Female	146 (13%)	147 (13%)
Male	1004 (87%)	1002 (87%)
Educational attainment*		
Less than primary	525 (46%)	539 (47%)
Primary and less than high school	452 (39%)	437 (38%)
At least high school	156 (14%)	154 (13%)
Other	14 (1%)	14 (1%)
Smoking status		
Never	159 (14%)	338 (29%)
Occasional	50 (4%)	88 (8%)
Regular		
Former	474 (41%)	458 (40%)
Current	467 (41%)	265 (23%)
Type of tobacco smoked†		
Blond tobacco only	92 (10%)	114 (16%)
Black tobacco only	383 (41%)	281 (39%)
Both types of tobacco	284 (30%)	194 (27%)
Unknown tobacco type	182 (19%)	132 (18%)

Unless otherwise stated, data are number of participants (%). *Information on education missing for three cases and five controls. †Defined only for regular smokers; information on type of tobacco missing for two controls.

Table 1: **Characteristics of study population**

Methods

Study population

The Spanish Bladder Cancer Study is a hospital-based case-control study based in 18 hospitals in five areas in Spain (Asturias, Barcelona metropolitan area, Vallès/Bages, Alicante, and Tenerife). Eligible "cases" were aged 21–80 years and had newly diagnosed, histologically confirmed carcinoma of the urinary bladder in 1998–2001. Diagnostic slides from each patient were reviewed by a panel of expert pathologists to confirm the diagnosis and to ensure uniformity of classification criteria, based on the 1998 system of WHO and the International Society of Urological Pathology.[36]

Controls were selected from patients admitted to participating hospitals with diagnoses thought to be unrelated to the exposures of interest, such as tobacco use. The distribution of reasons for hospital admission was: 37% hernias, 11% other abdominal surgery, 23% fractures, 7% other orthopaedic problems, 12% hydrocoele, 4% circulatory disorders, 2% dermatological disorders, 1% ophthalmological disorders, and 3% other diseases. Controls were individually matched to the cases for age at interview within 5-year categories, sex, ethnic origin, and region. Information on known or potential risk factors for bladder cancer for cases and controls was collected by means of computer-assisted personal interviews during the hospital admission. 84% of eligible cases and 88% of eligible controls agreed to take part in the study and were interviewed. Of the 1219 cases and 1271 controls interviewed, 1188 (97%) cases and 1173 (92%) controls provided a blood or buccal-cell sample for DNA extraction. Seven cases and 11 controls were excluded because of low amounts of DNA. To limit heterogeneity, 16 cases with neoplasias of non-transitional histology and six non-white individuals (five cases, one control) were excluded from the analyses. 15 individuals (seven cases, eight controls) with missing information on smoking status and seven (three cases, four controls) with DNA quality-control difficulties were also excluded from the analyses. Thus, the final study population available for analysis was 1150 cases and 1149 controls, all of whom were white.

Participants were classified as never smokers if they had smoked fewer than 100 cigarettes in their lifetime and ever smokers otherwise. Ever smokers were further classified as regular smokers if they had smoked at least one cigarette per day for 6 months or longer and occasional smokers otherwise. We defined current smokers as those regular smokers who had smoked within a year of the reference date; individuals who had smoked regularly but who had stopped smoking more than 1 year before the reference date were defined as former smokers. Most (81%) smokers of known tobacco type reported smoking black tobacco. In addition, the risks of bladder cancer in relation to the risk for never smokers were similarly raised among smokers of black tobacco alone, smokers of black and blond tobacco, and smokers of unknown tobacco type (data not shown). These subgroups were therefore combined as known or likely black-tobacco smokers. We obtained informed consent from potential participants in accordance with the National Cancer Institute and local institutional review boards.

Procedures

DNA for genotype assays was extracted from leucocytes with the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN, USA) for 1107 cases and 1032 controls included in the analyses. DNA from another 43 cases and 117 controls was extracted from mouthwash samples by a standard phenol–chloroform method. Genotype assays were done at the Core Genotyping Facility of the Division of Cancer Epidemiology and Genetics, National Cancer Institute, with the TaqMan (Applied Biosystems, Foster City, CA, USA), MGB Eclipse (Epoch Biosciences, Bothel, WA, USA), or MASSArray (Sequenom, San Diego, CA, USA) assay. A description of and methods for each specific assay can be found at the National Cancer Institute SNP500Cancer website.[37] Genotype assays were done for *NAT1* (Ex1-88A>T rs1057126, Ex1-81A>C rs15561, V149I rs4987076, R187Q rs4986782, R187* rs5030839, R33*, D251V, R64W), *NAT2* (K268R rs1208, G286E rs1799931, R64Q rs1801279, Y94Y rs1041983, I114T rs1801280, L161L rs1799929, R197Q rs1799930), *GSTM1* deletion (SNP500Cancer ID:GSTM1-02), *GSTT1* deletion (SNP500Cancer ID:GSTT1-02), *GSTP1* (I105V rs947894, A114V), and *GSTM3* (V224I rs7483, IVS7 –30G>T rs1537234). All genotypes studied were in Hardy-Weinberg equilibrium among the control population. Duplicate quality-control samples showed 100% agreement for all but four assays (range 98·2% to 99·6%).

Information from the *NAT1* and *NAT2* single-nucleotide polymorphisms analysed in this study was used to assign the most likely *NAT1* and *NAT2* alleles previously identified in human populations.[38,39] Individuals homozygous for *NAT2* rapid-acetylator alleles (*NAT2*4, NAT2*11A, NAT2*12A, NAT2*12B, NAT2*12C, NAT2*13*) were classified as rapid-acetylator phenotype; individuals homozygous for slow-acetylator alleles were classified as slow-acetylator phenotype, and heterozygous individuals (one rapid and one slow *NAT2* allele) were classified as intermediate-acetylator phenotype. Individuals with missing information for four rare *NAT1* single-nucleotide polymorphisms (R187*, R33*, D251V, and R64W with more than 99% homozygous wild-type individuals) were assumed to be homozygous for *NAT1*4*. On the basis of previous studies, the *NAT1*10* allele was deemed to be the "at risk" allele. *GSTM1* genotypes were defined as null (–/–) if a deletion was present in both copies of the gene and present if one (+/–) or none (+/+) of the copies had a deletion. The two *GSTP1* (I105V and A114V) and *GSTM3* (V224I and IVS7 –30G>T) polymorphisms investigated were in strong linkage disequilibrium ($D'=1\cdot0$, $R^2=0\cdot10$ and $D'=1\cdot0$, $R^2=0\cdot68$, respectively). Participants were classified according to the presence of three *GSTP1* variants that have been found to encode functionally differing *GSTP1* proteins: *GSTP1*A* (105 Ile; 114 Ala), *GSTP1*B* (105 Val; 114 Ala), and *GSTP1*C* (105 Val; 114 Val).[40]

Statistical analysis

Odds ratios, as measure of relative risk, and 95% CI were estimated from logistic regression models, with adjustment for sex, age at interview, region, and smoking status (never, occasional, former, or current). These unconditional models provided estimates similar to those from conditional logistic regression models for individually matched pairs. Interactions between genotypes and smoking habits were also investigated by the semiparametric maximum likelihood estimator method (SPMLE)[41] to allow estimation of parameters under the assumption of genotype–smoking and genotype–sex independence in the source population. This assumption is supported by strong evidence from previous studies for independence of *NAT2* and *GSTM1* genotypes from cigarette smoking[8,11,42] and sex[43] in the control populations. Tests for multiplicative interaction were used to assess whether the genotype odds ratios within categories of smoking habits differed significantly from each other, or whether smoking odds ratios within genotype categories differed significantly from each other. When no multiplicative interactions were present, we also tested for additive interactions, because departures from the additive model can exist in the absence of multiplicative interactions and they might have biological implications under certain biological models.[44] The synergy index was used as a measure of additive interaction and its CI was calculated by use of previously published formulae.[45]

We updated previous meta-analyses on *NAT2*, *GSTM1*, and bladder cancer and used similar selection criteria for studies—ie, case-control studies in the general population.[4,8,11] Relevant studies published up to February, 2005, were identified in a MEDLINE search. For studies of *NAT2*[4,11] and *GSTM1*[8] included in previously published meta-analyses, we used data from

Genotype	Cases	Controls	Odds ratio (95% CI)	p
NAT2*				
Rapid	55	66	1·0	
Intermediate	351	427	1·0 (0·7–1·5)	0·97
Slow	728	637	1·4 (0·9–2·1)	0·10
Slow *vs* rapid/intermediate			1·4 (1·2–1·7)	0·0002
***GSTM1*†**				
+/+	70	107	1·0	
+/–	352	454	1·2 (0·8–1·7)	0·38
–/–	716	571	1·9 (1·4–2·7)	0·0002
Null *vs* present			1·7 (1·4–2·0)	<0·0001
NAT1				
*NAT1*4/NAT1*4*	585	574	1·0	
*NAT1*10/NAT1*4*	327	326	1·0 (0·8–1·2)	0·62
*NAT1*10/NAT1*10*	53	42	1·2 (0·8–1·8)	0·48
***GSTT1*‡**				
+/+	327	340	1·0	
+/–	572	533	1·2 (1·0–1·5)	0·05
–/–	230	248	1·0 (0·8–1·3)	0·90
***GSTP1* I105V**				
Ile/Ile	486	488	1·0	
Ile/Val	525	531	1·0 (0·8–1·2)	0·93
Val/Val	130	119	1·2 (0·9–1·5)	0·35
***GSTP1* A114V§**				
Ala/Ala	966	917	1·0	
Ala/Val	113	85	1·3 (1·0–1·8)	0·07
Val/Val	4	5	0·9 (0·2–3·4)	0·85
***GSTP1* I105V/A114V combination¶**				
*GSTP1*A/GSTP1*A*	456	441	1·0	
*GSTP1*A/GSTP1*B*	409	402	1·0 (0·8–1·2)	0·92
*GSTP1*B/GSTP1*B*	95	69	1·4 (1·0–1·9)	0·09
*GSTP1*C*/any other variant	113	90	1·3 (0·9–1·8)	0·12
***GSTM3* V224I**				
Val/Val	565	588	1·0	
Val/Ile	472	451	1·1 (0·9–1·3)	0·30
Ile/Ile	92	88	1·0 (0·7–1·4)	0·89
***GSTM3* IVS7 –30G>T**				
GG	439	464	1·0	
GT	529	504	1·1 (0·9–1·4)	0·19
TT	160	154	1·1 (0·8–1·4)	0·64

Odds ratios from conventional logistic regression models adjusted for sex, age, region, and smoking status. Missing information on *NAT2* for 16 cases *vs* 19 controls; on *NAT1* for 123 *vs* 124, including individuals with rare or undeterminable alleles (62 *vs* 83 with other *NAT1* genotypes are not shown); on *GSTM1* for 11 *vs* 17; on *GSTT1* for four *vs* 12; on *GSTP1* I105V for nine *vs* 11; on *GSTP1 A114V* for 24 *vs* 25; on *GSTM3 V224I* for 21 *vs* 22; and on *GSTM3 IVS7 –30G>T* for 22 *vs* 27. *The proportions of *NAT2* slow acetylators among cases with superficial tumours (Ta) grade 1, grade 2, and grade 3, tumours involving the submucosa (T1) grades 2/3, tumours infiltrating muscle (T2) grades 2/3, and metastatic tumours (T3/T4) were 64%, 65%, 65%, 67%, 61%, and 64% (p=0·72, p=0·80, p=0·55, p=0·80, p=0·94, respectively, compared with Ta/grade 1 and adjusted for age, region, and smoking status). †*GSTM1* +/+ and +/– could not be distinguished for one case, who contributed to the estimation of odds ratio for *GSTM1* present *vs* null genotypes. The proportions of *GSTM1* null genotype among cases with superficial tumours Ta/grade 1, Ta/grade 2, and Ta/grade 3, tumours involving the submucosa (T1) grades 2/3, tumours infiltrating muscle (T2) grades 2/3, and metastatic tumours (T3/T4) were 61%, 62%, 61%, 67%, 61%, and 66% (p=0·79, p=0·93, p=0·14, p=0·80, and p=0·35, respectively, compared with Ta/grade 1 and adjusted for age, region, and smoking status). ‡*GSTT1* +/+ and +/– could not be distinguished for 17 cases and 16 controls. §Assay done only among cases and controls with blood DNA (96% of cases and 90% of controls). ¶Classified according to Ali-Osman and colleagues[40] to reflect three functionally different *GSTP1* variants: *GSTP1*A* (105 Ile; 114 Ala), *GSTP1*B* (105 Val; 114 Ala), and *GSTP1*C* (105 Val; 114 Val).

Table 2: **Odds ratios for the associations of polymorphisms in *NAT* and *GST* genes and bladder-cancer risk**

Smoking characteristics	Frequency				Odds ratio (95% CI) for *NAT2* slow genotype association by smoking characteristic	Odds ratio (95% CI) for joint *NAT2* slow genotype and smoking association		p*
	NAT2 rapid/intermediate		*NAT2* slow			*NAT2* rapid/intermediate	*NAT2* slow	
	Cases	Controls	Cases	Controls				
Smoking status†								
Never	66	131	91	199	0·9 (0·6–1·3)	1·0	0·9 (0·6–1·3)	
Ever	340	362	637	438	1·6 (1·3–1·9)	2·9 (2·0–4·2)	4·6 (3·2–6·6)	0·008
Occasional	16	37	32	48	1·4 (0·6–2·9)	1·2 (0·6–2·4)	1·6 (0·9–2·9)	0·28
Former	161	212	310	240	1·7 (1·3–2·2)	2·4 (1·6–3·7)	4·1 (2·8–6·1)	0·006
Current	163	113	295	150	1·4 (1·1–2·0)	5·2 (3·4–8·0)	7·5 (5·0–11·3)	0·05
Type of tobacco‡								
Never	66	131	91	199	0·9 (0·6–1·3)	1·0	0·9 (0·6–1·3)	
Black	284	272	553	328	1·6 (1·3–2·0)	3·6 (2·4–5·4)	5·9 (4·0–8·7)	0·005
Blond	40	52	52	61	1·2 (0·7–2·1)	2·5 (1·4–4·3)	2·9 (1·7–4·9)	0·36
Smoking intensity§								
Never	66	131	91	199	0·9 (0·6–1·3)	1·0	0·9 (0·6–1·3)	
<10	26	55	43	61	1·7 (0·9–3·2)	0·6 (0·3–1·1)	0·9 (0·5–1·8)	0·09
10–19	67	57	106	77	1·2 (0·7–1·9)	1·3 (0·7–2·6)	1·6 (0·9–3·0)	0·31
20–29	143	108	263	133	1·4 (1·0–2·0)	1·6 (0·9–2·9)	2·3 (1·3–4·1)	0·05
30–39	31	27	88	42	1·8 (0·9–3·5)	1·4 (0·6–3·0)	2·5 (1·3–4·8)	0·06
≥40	54	73	102	71	1·7 (1·1–2·8)	1·0 (0·5–2·0)	1·8 (0·9–3·3)	0·03

*For differences between the odds ratio for *NAT2* slow-acetylation genotype within strata defined by smoking characteristics compared with never smokers. This test is equivalent to testing whether the observed joint odds ratio for *NAT2* slow-acetylator genotype and smoking characteristics differs from the product of the odds ratio for *NAT2* slow genotype among never smokers and the smoking characteristic among *NAT2* rapid/intermediate genotype. †Odds ratios are from conventional logistic regression models adjusted for sex, age, and region. ‡The p for interaction for former *vs* current smokers is 0·44 and for blond *vs* black tobacco is 0·33. Odds ratios are from conventional logistic regression models adjusted for sex, age, region, and smoking cessation (former/current). Black is for known or likely black tobacco smokers. §Cigarettes per day. Odds ratios are from conventional logistic regression models adjusted for age, sex, region, smoking duration (<20 years, 20–29 years, 30–39 years, 40–49 years, ≥50 years), and smoking cessation (current/former). Odds ratios for *NAT2* slow acetylation for different categories of smoking intensity did not differ significantly from the highest intensity category (p for interaction for categories <10, 10–19, 20–29, 30–39 cigarettes per day compared with ≥40 cigarettes per day are 0·96, 0·30, 0·58, and 0·90 respectively for total intensity).

***Table 3:* NAT2 slow-acetylation genotype, smoking characteristics, and bladder-cancer risk**

those papers rather than the data from the original reports, with a few exceptions: for Taylor (1998) in Marcus and colleagues' meta-analysis,[4,11] and for Lin (1994) and Bell (1993) in Engel and colleagues' meta-analysis[8] we used the original report to distinguish between black and white individuals; for Horai (1989) and Karakaya (1986) in Marcus and colleagues' paper,[11] we recalculated odds ratios and 95% CI to obtain exact estimates. For studies not included in previous meta-analyses that did not present crude odds ratios and 95% CI, we calculated them from published data.

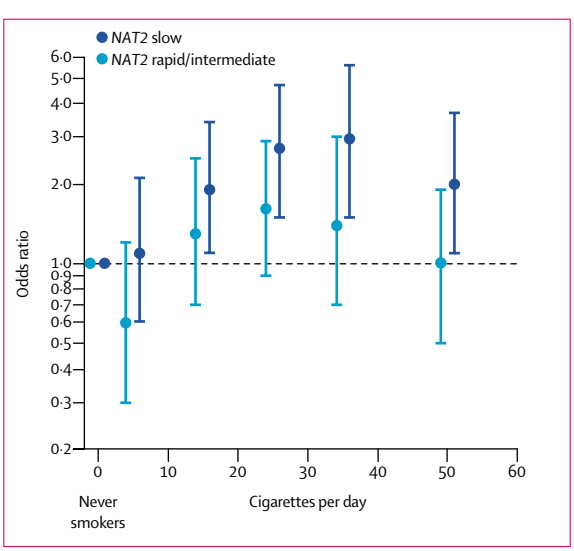

***Figure 1:* Association between smoking intensity (average number of cigarettes per day in categories of 10 cigarettes) and bladder-cancer risk compared with never smokers, stratified by *NAT2* acetylation genotype**
Odds ratios are from conventional logistic regression models adjusted for age, sex, region, smoking duration (<20 years, 20–29 years, 30–39 years, 40–49 years, ≥50 years), and smoking cessation (current/former smokers). Error bars represent 95% CI. p values for interaction are shown in table 3.

Random-effects summary measures were calculated by weighting of each study result by a factor of within-study and between-study variance.[46] Homogeneity of study results was assessed by the *Q* statistic, and publication bias was assessed by Begg's[47] and Egger's tests.[48] A case-only design[49] was used in meta-analyses to assess the presence of a multiplicative interaction between *NAT2* and *GSTM1* genotypes and smoking status (ever/never) because that approach meant we could include some studies without information on the cross-classification of genotype and smoking status among controls, it removed possible biases resulting from the inclusion of hospital controls with diseases related to tobacco use, and it is a powerful design to test for multiplicative interactions under the assumption of independence of *NAT2* and *GSTM1* from smoking status in the population. Statistical analyses were done with STATA (version 8.2, special edition).

Role of the funding source

The study sponsors had no role in the design of the study; in the collection, analysis, or interpretation of the data; or in the writing of the report. The corresponding

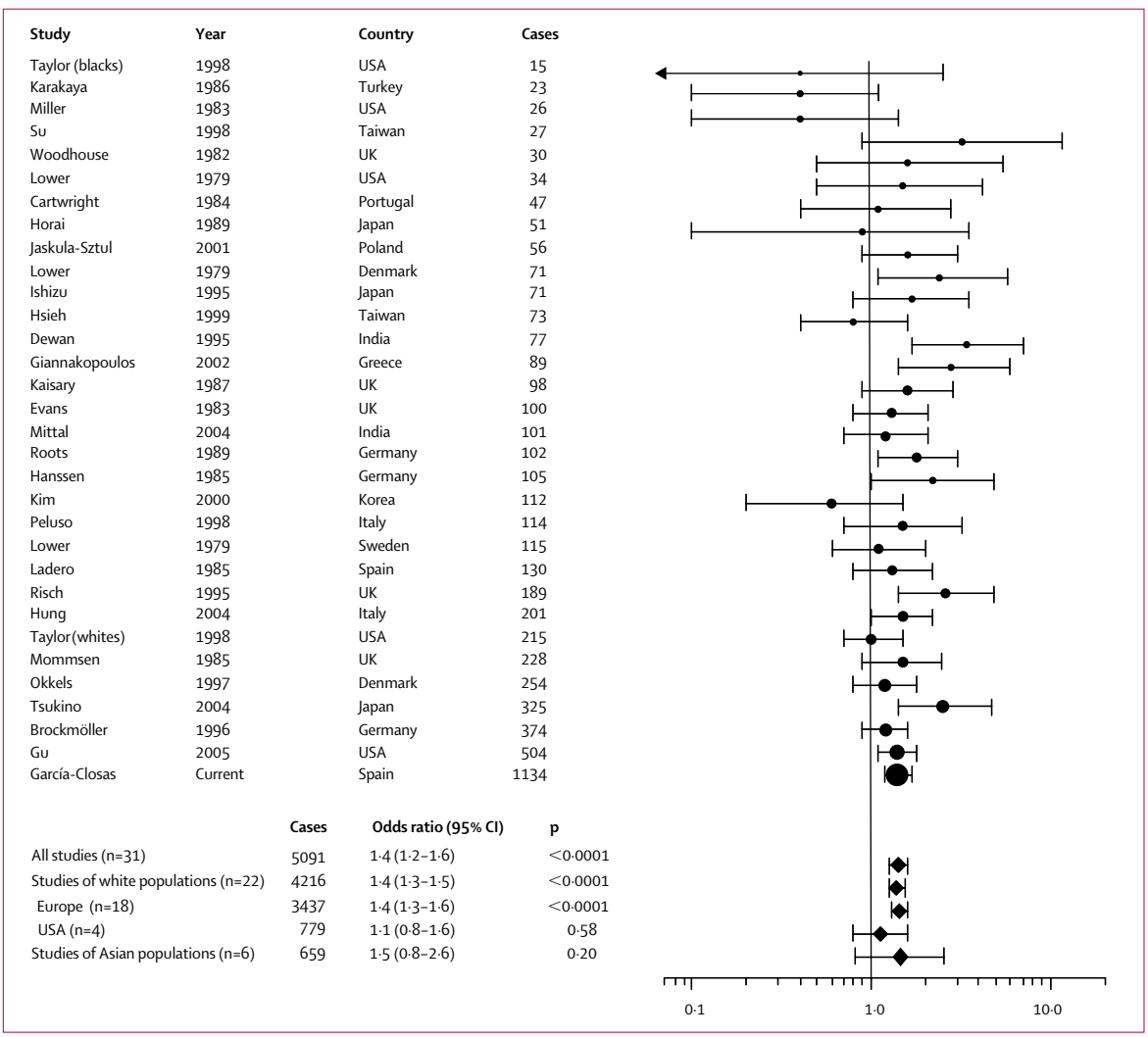

Figure 2: **Meta-analysis of studies of *NAT2* slow-acetylation genotype and bladder-cancer risk**
Numbers of cases are individuals with *NAT2* information.

author had full access to all the data in the study and had final responsibility for the decision to submit the paper for publication.

Results

The study population was white, predominantly male, and a high proportion were smokers, mostly of black tobacco (table 1). In this population, *NAT2* slow-acetylator and *GSTM1* null (–/–) genotypes significantly increased the risk of bladder cancer (table 2). The risk of bladder cancer was 40% higher in *NAT2* slow acetylators than in *NAT2* rapid or intermediate acetylators (odds ratio 1·4 [95% CI 1·2–1·7]); *NAT2* rapid acetylators and intermediate acetylators had similar risks of bladder cancer (table 2). The odds ratios for bladder cancer for individuals with deletion of one or two copies of the *GSTM1* gene were 1·2 (0·8–1·7) and 1·9 (1·4–2·7), respectively (trend test $p<0·0001$). Individuals with the null genotype had a 70% higher risk of bladder cancer than those with one or two copies of the *GSTM1* gene (table 2). The associations for *NAT2* and *GSTM1* genotypes were similar irrespective of tumour grade or stage (table 2), and there was no evidence that these associations differed by age or sex (data not shown).

The joint association for the combined *NAT2* slow-acetylator and *GSTM1* null genotype, present in 28% of the control population, compared with *NAT2* rapid/intermediate-acetylator and *GSTM1* present genotype (odds ratio 2·2 [1·7–2·9]) was consistent with a weak multiplicative interaction between these two genetic variants; however, the test for multiplicative interaction was not significant ($p=0·15$). None of the other genetic polymorphisms investigated was significantly associated with an increased risk of bladder cancer (table 2), and there was no evidence of multiplicative interactions between them (data not shown).

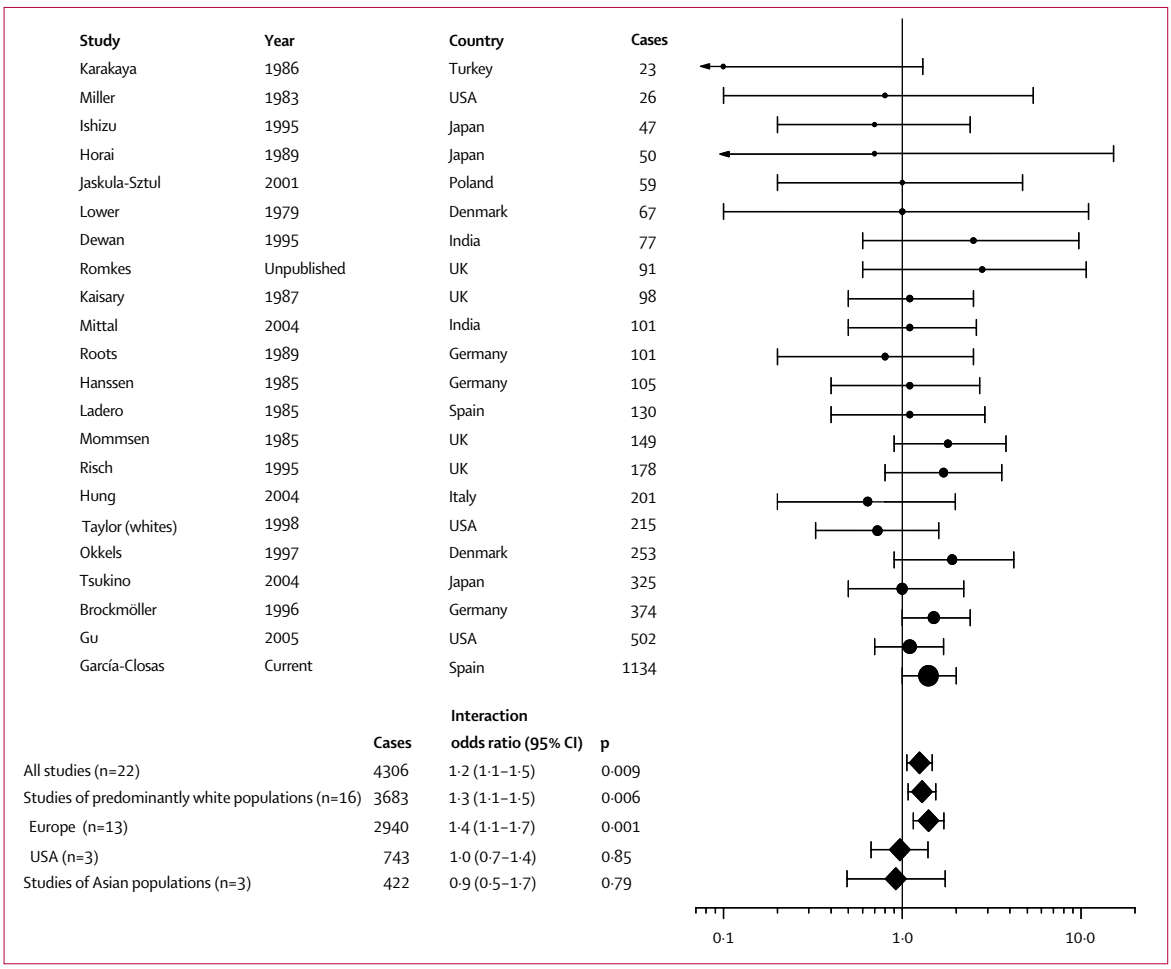

Figure 3: **Case-only meta-analysis of studies of *NAT2* slow-acetylation genotype, cigarette smoking, and bladder-cancer risk**
Numbers of cases are individuals with *NAT2* and smoking information.

Conventional logistic regression analyses showed a significant multiplicative interaction between *NAT2* slow acetylation and cigarette smoking status (ever/never p=0·008; table 3) with an interaction odds ratio of 1·8 (1·2–2·8). The evidence for a multiplicative interaction was somewhat weaker (interaction odds ratio 1·4 [1·0–1·9], p=0·08) when we used SPMLE logistic regression, which assumed genotype–smoking and genotype–sex independence conditional on age, in the source population. Estimates for the *NAT2* slow-acetylation association with bladder cancer were similar for occasional, current, and former smokers (table 3). The data suggested that the association of *NAT2* slow-acetylation genotype with bladder cancer was stronger for known or likely smokers of black tobacco than for smokers of blond tobacco (table 3). However, this difference was not significant (table 3). The *NAT2* and smoking intensity interaction is described by showing the odds ratios for *NAT2* slow acetylation genotype by smoking intensity (table 3), for the joint association of *NAT2* slow genotype and smoking intensity (table 3), and for smoking intensity by *NAT2* acetylation genotype (figure 1). *NAT2* slow acetylators were at a higher risk from cigarette smoking than rapid or intermediate acetylators, for all smoking intensities (figure 1). The magnitude of the association between *NAT2* slow acetylation and bladder-cancer risk among regular smokers was similar across different smoking intensities (table 3), durations, and pack-years (data not shown). As with the interaction between *NAT2* and smoking status, SPMLE odds ratios and p values for interactions with other smoking characteristics were slightly attenuated compared with conventional analyses (data not shown).

Neither conventional nor SPMLE logistic regression showed a significant multiplicative interaction (odds ratio 0·7 [0·4–1·1], p=0·09, and 0·8 [0·5–1·1], p=0·15, respectively) for the association of *GSTM1* null and smoking status (ever/never) on bladder-cancer risk. Thus, the relative risk of bladder cancer for *GSTM1* null compared with present genotype does not vary by smoking status. No multiplicative interactions were found for other smoking characteristics such as smoking

cessation (current *vs* former smokers), smoking intensity, or duration (data not shown). Since an additive interaction can exist in the absence of a multiplicative interaction, and departures from the additive model might have biological implications under certain assumptions, we then tested for an additive interaction. Both conventional and SPMLE logistic regressions showed significant departures from the additive model (ie, additive interactions) or *GSTM1* null genotype and smoking status, with synergy indices of 1·3 (95% CI 1·0–1·6; p=0·04) and 1·4 (1·1–1·7; p=0·001), respectively.

We updated a previously published meta-analysis of 22 studies of *NAT2* and bladder cancer[4] to include data from our study and eight additional studies,[17–19,27,28,34,50,51] including a total of 5091 cases and 6501 controls (figure 2). The summary relative risk for *NAT2* slow acetylators compared with rapid/intermediate acetylators was 1·4 (1·2–1·6; p<0·0001) with no evidence for publication bias according to Begg's (p=0·94) or Egger's tests (p=0·91). There was some evidence of study heterogeneity (*Q* test p=0·04), which was not present when 15 studies with fewer than 100 cases each were excluded (summary odds ratio 1·4 [1·2–1·5]; *Q* test p=0·31). Summary estimates for white populations (56% prevalence of *NAT2* slow acetylators in controls) and Asian populations (11% prevalence of *NAT2* slow acetylators in controls) were similar (p=0·87; figure 2). The summary relative risk for studies of white populations in the USA was lower than that for studies done in Europe, which accounted for most (82%) white cases; however this difference was not significant (p=0·18; figure 2).

We also updated a case-only meta-analysis of *NAT2* and smoking interaction on bladder-cancer risk[11] to include results from our study and five additional studies published after the meta-analysis[17,19,34,50,51] (figure 3). This analysis included a total of 4306 cases and showed

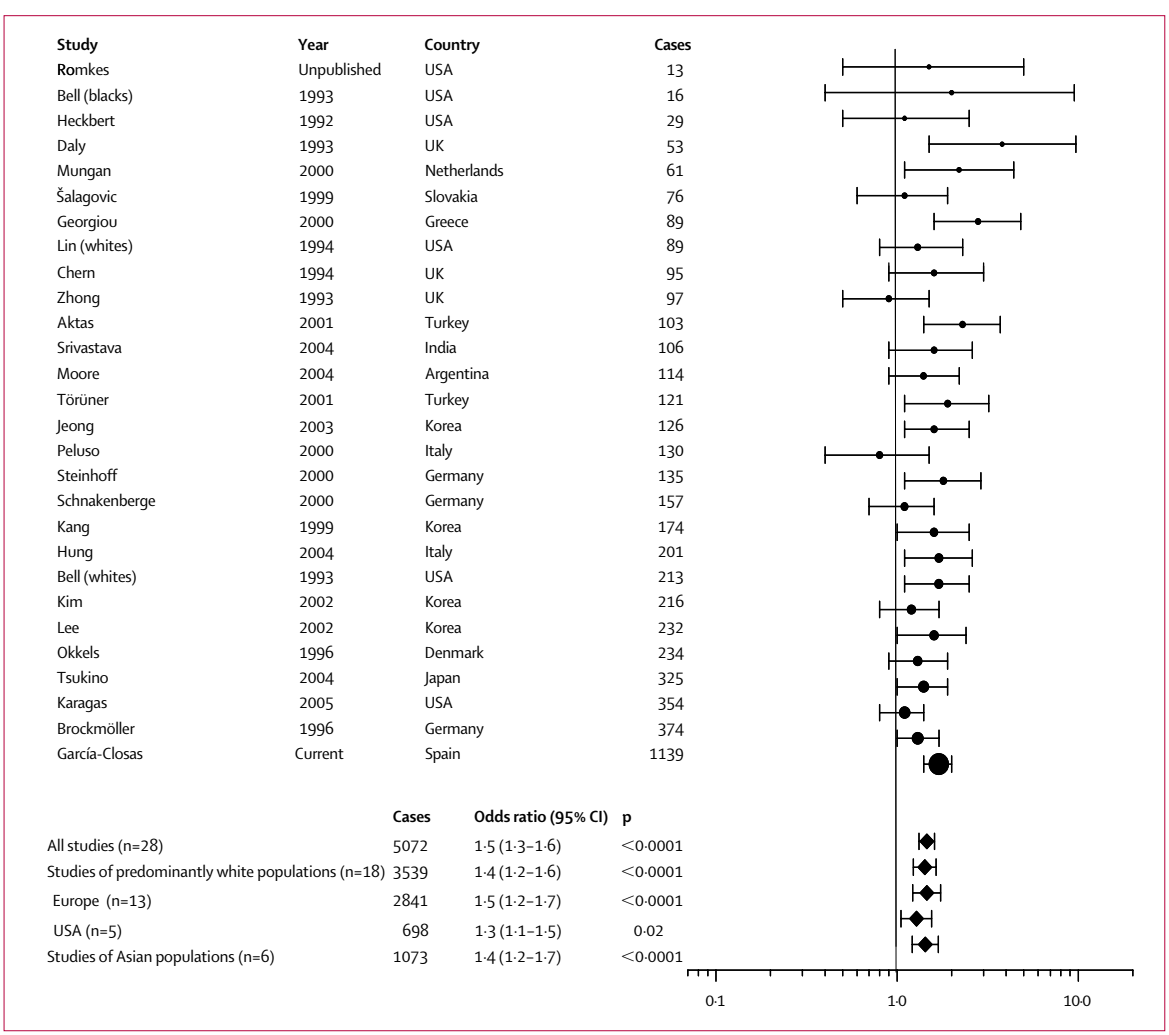

Figure 4: **Meta-analysis of studies of *GSTM1* null genotype and bladder-cancer risk**
Number of cases for studies in Engel et al[8] are based on table 1 of that paper.

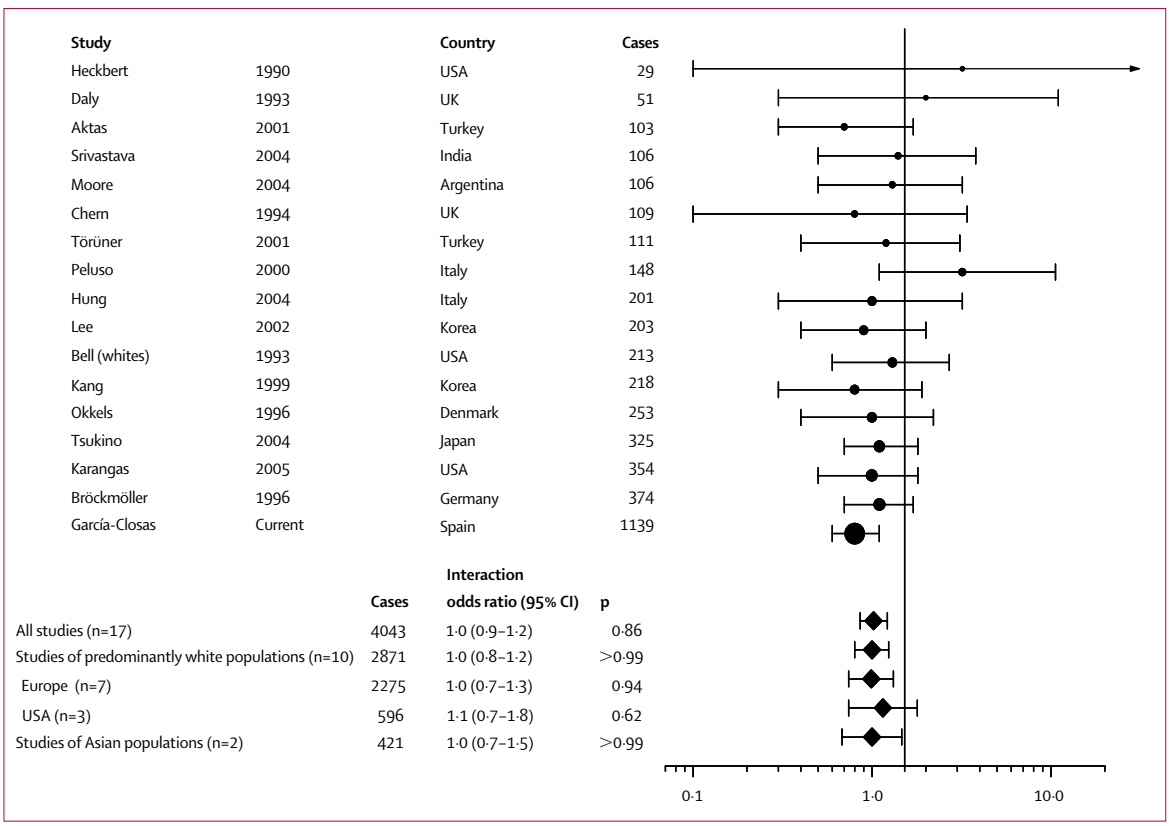

Figure 5: **Case-only meta-analysis of studies of *GSTM1* null genotype, cigarette smoking, and bladder cancer**
Numbers of cases are individuals with *GSTM1* and smoking information. Numbers of cases in studies included in Engel et al[8] are based on data used for the pooled analyses published in that paper.

evidence for an interaction with a summary estimate of 1·2 (1·1–1·5; p=0·009) for all populations combined. There was no evidence of overall study heterogeneity (*Q* test p=0·84) or publication bias (Begg's test p=0·40, Egger's test p=0·13). The point estimate for interaction was higher in white than in Asian populations (1·3 *vs* 0·9) and in European than in US white populations (1·4 *vs* 1·0); however, these differences were not significant (p=0·32 and 0·09, respectively; figure 3).

A meta-analysis of 17 studies of *GSTM1*[8] was also updated to include our study, ten additional studies,[17,21,22,24,26,29,30,35,52,53] and an update from a previously published study,[50] yielding a total of 5072 cases and 6466 controls (figure 4). The summary odds ratio for *GSTM1* null versus present genotype for all populations combined was 1·5 (1·3–1·6; p<0·0001) with no evidence of study heterogeneity (*Q* test p=0·10) or publication bias by Begg's test (p=0·27) or Egger's test (p=0·57). Summary estimates were similar and significant in white populations (51% of *GSTM1* null genotype in controls) and Asian populations (53% of *GSTM1* null genotype in controls), as well as in US and European white populations (figure 4).

An updated case-only meta-analysis of studies that investigated the *GSTM1*-smoking interaction[8] to include our study and seven other studies[17,21,22,29,30,35,52] (17 studies of 4043 cases) confirmed the absence of a multiplicative interaction with a summary odds ratio of 1·0 (0·9–1·2; p=0·86; figure 5). The *Q* test showed no evidence of study heterogeneity (p=0·87), and Begg's test (p=0·15) and Egger's test (p=0·03) suggested the presence of publication bias. Summary estimates for the interaction were very similar for all population subgroups (figure 5).

Discussion

This report provides compelling evidence of an increased bladder-cancer risk associated with the *GSTM1* null and *NAT2* slow-acetylation genotypes. The latter association was particularly important among cigarette smokers. Although the relative risks for polymorphisms in *NAT2* and *GSTM1* genes are modest, these polymorphisms could account for a large proportion of bladder cancers because they are very common in the population. From our data, we estimate that these polymorphisms cause 31% (95% CI 20–46) of bladder cancers in white populations. In addition, we provide strong evidence against a substantial overall association for polymorphisms in other *NAT* and *GST* genes, with the possible exception of small to moderate associations for the *NAT1*10/*10* and *GSTP1 114Val/Val* genotypes.

The new meta-analysis of studies of *NAT2* slow acetylation and bladder-cancer risk showed that this association is robust and similar for white and Asian populations. The lack of significance for the association in Asian populations might be explained by substantially lower statistical power to detect associations in Asian studies owing to a lower prevalence of *NAT2* slow acetylators (11% for Asian *vs* 56% for white populations), along with a smaller number of cases available for the meta-analysis. We also found that *NAT2* slow acetylators are especially susceptible to the adverse effects of cigarette smoking on bladder-cancer risk. This gene–environment interaction has strong biological plausibility, because *NAT2* slow acetylators have decreased capacity to detoxify aromatic monoamines by N-acetylation,[13] tobacco smoking is a primary source of exposure to aromatic amines in the general population, and aromatic amines are suspected to be the primary bladder carcinogen in tobacco smoke.[12] Our data suggest that *NAT2* slow acetylation does not increase bladder-cancer risk among never smokers, although they do not rule out a small increase in risk in this group.

Because the content of aromatic amines is higher in black than in blond tobacco,[54] the effect of *NAT2* slow acetylation could conceivably be stronger for smokers of black tobacco. Our data are consistent with this hypothesis, although the differences were not significant. The magnitude of the association between *NAT2* slow acetylation and bladder-cancer risk was similar for different smoking intensities in our study population. Our meta-analysis of the interaction between smoking status and *NAT2* slow-acetylation genotype suggested a stronger interaction with ever/never smoking in European than in US studies (p=0·09). This difference could result from the lower content of aromatic amines in blond tobacco, which is generally smoked in the USA, than in the black tobacco commonly smoked in parts of Europe. This explanation is consistent with a study of a population in the USA that found an interaction between *NAT2* slow-acetylation genotype and smoking only for heavy smokers.[34]

Distinction of individuals with one and two copies of the *GSTM1* gene, an issue that has not been adequately addressed in previous studies of bladder cancer, suggests the presence of a gene-dosage effect with relative risks of 1·2 (0·8–1·7) and 1·9 (1·4–2·7) for individuals with one or no copies of *GSTM1*, respectively, compared with those with two copies (p for trend <0·0001). Meta-analyses of the association between the deletion of two copies of the *GSTM1* gene (null genotype) compared with the presence of one or two copies (present genotype), as calculated from previous studies that could not distinguish between these two groups of individuals, showed that this association is robust (p<0·0001) and similar in magnitude and significant across different population subgroups.

The relative risk for *GSTM1* null genotype and bladder cancer was similar for smokers and never smokers in our study population and in meta-analysis within population subgroups. This finding suggests the presence of an additive interaction, which is supported by our data (p=0·04). This observation is compatible with equal protection by *GSTM1* activity against tobacco-related and non-tobacco-related bladder cancers. This finding suggests that *GSTM1* lowers the risk of bladder cancer through mechanisms that are not specific to the detoxification of polycyclic aromatic hydrocarbons in tobacco smoke. Other mechanisms of action for *GSTM1* could be protection from oxidative damage through metabolism of reactive oxygen species.[55] Our data did not confirm previously suggested differences in risk for *NAT2* slow-acetylation and *GSTM1* null genotypes by tumour grade or stage at presentation.[26,56–59] Our findings are consistent with a potential interaction between *NAT2* slow-acetylation and *GSTM1* null genotypes; however, further evidence is needed to confirm this interaction.

Associations between bladder-cancer risk and polymorphisms in genes encoding the NAT1 enzyme involved in the activation of aromatic amines by O-acetylation,[13] and other GST enzymes that have important roles in the detoxification of polycyclic aromatic hydrocarbons and other carcinogens[60] have been less fully explored. Previous studies have provided inconsistent evidence for an association between bladder-cancer risk and *NAT1*10* alone or in combination with *NAT2* slow acetylation,[14–19,34] *GSTT1* null alone or in combination with *GSTM1* null genotype,[17,20–31,35] and *GSTP1 105 Val/Val* genotype.[17,21,32,33] The data from our study do not support a substantial association between *GSTT1* and *GSTM3* genotypes and bladder-cancer risk. We found no significant increases in bladder-cancer risk associated with polymorphisms in *NAT1* or *GSTP1* genes; however, our estimates did not exclude a small to moderate association for the *NAT1*10/*10* genotype compared with the *NAT1*4/*4* genotype or for genotypes with the *GSTP1* 114Val allele compared with the 114Ala/Ala genotype.

Analyses by conventional logistic regression suggested a modification of the association between risk of bladder cancer and *NAT2, GSTM1,* and *NAT1* genotypes by sex. However, the modifications by sex were explained by unexpected differences in the genotype distribution for male and female controls.

Our study had several strengths: high participation rates, large sample size, and high-quality information on exposure and genotype. Specifically, we made an effort to improve the precision in genotype estimation by genotyping the seven single-nucleotide polymorphisms in *NAT2* that probably account for virtually all genetic variation in white populations,[61] and we developed assays that successfully distinguished individuals with one or two copies of the *GSTM1* and *GSTT1* genes. We also used the SPMLE method[41] to increase power and reduce bias in the estimation of interactions, because of the strong

evidence from previous studies for independence of *NAT2* and *GSTM1* genotypes from cigarette smoking status[8,11,42] and sex[43] in the general population. To limit selection bias, we carefully selected controls from patients admitted for various diagnoses that were thought to be unrelated to exposures of interest, including tobacco use. Genotype frequencies among the control population were similar to those previously reported. We found no significant overall differences in genotype frequencies across control diagnoses that could have biased our results.

Although this study is the largest to date on the role of genetic polymorphisms and bladder-cancer risk and had adequate statistical power to detect modest genotype associations, the power to detect interactions was limited. Meta-analyses including previous studies improved our ability to make inferences on interactions, when there was an adequate number of previous studies with homogeneous results. A consortium of bladder-cancer studies has been formed to facilitate the pooling of comparable data on environmental and genetic risk factors across studies that will help overcome the limited power of individual studies to investigate complex interrelations.

Contributors

M García-Closas, N Malats, D Silverman, M Dosemeci, M Kogevinas, F X Real, and N Rothman participated in the study design, enrolment of patients, and gene selection. G Castaño-Vinyals, M Torà, F Fernández, C Samanic, A Tardón, C Serra, A Carrato, and R García-Closas participated in the study design and enrolment of patients. D W Hein, M Yeager, R Welch, and S Chanock participated in gene selection and genotyping. J Lloreta participated in the pathology review. N Chatterjee and S Wacholder participated in the statistical analyses. M García-Closas did the statistical analyses and drafted the paper with input from all investigators.

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Conflict of interest statement

We declare that we have no conflict of interest.

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